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# Identification of the major sites of autophosphorylation of the murine protein-tyrosine kinase Syk

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**Abstract**

The protein tyrosine kinase p72$^{syk}$ (Syk) is expressed in a variety of hematopoietic cell types, including B cells, thymocytes, mast cells and others. Both the activity and phosphotyrosine content of this enzyme increase in these cells in response to engagement of the appropriate cell surface receptors. Herein, we describe the cloning of murine Syk and its expression in Sf9 cells as a catalytically active protein. Full-length Syk and a catalytically active 42.5 kDa carboxyl terminal fragment were also expressed as glutathione S-transferase fusion proteins. Comparative reverse phase HPLC and 40% alkaline gel analysis of tryptic digests of phosphorylated Syk demonstrated that all of the major sites of autophosphorylation were also present in GST-Syk and all but one were contained in the 42.5 kDa fragment. The sites of autophosphorylation were identified using a combination of Edman sequencing and mass spectrometric analysis. Ten sites were identified. One site is located in the amino terminal half of the molecule between the two tandem Src homology 2 (SH2) domains. Five sites are located in the hinge region located between the carboxyl terminal SH2 domain and the kinase domain. Two sites lie in the kinase domain within the catalytic loop and two near the extreme carboxyl terminus. Sequences of phosphorylation sites located within the hinge region predict that Syk serves as a docking site for other SH2 domain-containing proteins. Consistent with this prediction, autophosphorylated Syk efficiently binds the carboxyl terminal SH2 domain of phospholipase C-$\gamma$1.

*Keywords:* Protein phosphorylation; Lymphocyte activation; Protein-tyrosine kinase; Tyrosine phosphorylation; Antigen receptor

## 1. Introduction

Protein tyrosine kinase (PTKs) catalyze the transfer of phosphate from ATP to the hydroxyl groups of tyrosine residues on substrate proteins. Most PTKs also catalyze inter- or intramolecular autophosphorylation reactions that lead to their own covalent modification. These autophosphorylations play important functional roles. For many cytosolic and transmembrane PTKs, autophosphorylation of residues within the active site is required for maximal kinase activity [1–5]. For growth factor receptor kinases, this autophosphorylation-dependent activation occurs as receptors dimerize after interacting with their specific extracellular ligands [6,7]. Autophosphorylation also generates binding sites for important signaling molecules that bind phosphotyrosine

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residues via 100 amino acid stretches known as Src Homology 2 (SH2) domains [8]. This phenomenon has been best characterized for the platelet-derived growth factor receptor where ligation results in the autophosphorylation of several tyrosine residues that serve as binding sites for SH2 domain-containing proteins such as c-Src, phosphatidylinositol 3-kinase (PI3-Kinase), phospholipase C-$\gamma$ (PLC-$\gamma$), rasGAP, protein tyrosine phosphatase 1D (Syp) and others [9]. Many of these proteins subsequently serve as substrates for the activated receptor.

In contrast to growth factor receptors, antigen receptors on hematopoietic cells lack intrinsic kinase activity, yet protein-tyrosine phosphorylation plays an important role in signal transduction through these receptors. Receptors such as the B cell antigen receptor (BCR) function by virtue of their physical and functional associations with cytoplasmic, Src- and non-Src-family PTKs. Aggregation of the BCR results in a marked increase in the tyrosine phosphorylation of several cellular proteins including several SH2 domain-containing proteins such as PLC-$\gamma$, PI3-kinase, rasGAP, SHC and others [10]. We [11] and others [12,13] have demonstrated that the BCR associates with the cytosolic PTK, $p72^{syk}$ (Syk). Both the kinase activity of Syk and its phosphotyrosine content increase in response to receptor-mediated B cell activation [12–15]. In Syk-deficient mice, B cells fail to develop properly, most likely due to defective signaling from the pre-BCR [16]. It is likely that Syk mediates the coupling of the receptor to downstream signaling molecules since receptor-mediated phosphorylation of PLC-$\gamma$2 is attenuated in a Syk-deficient avian B cell line [17] and the generation of $IP_3$ is blocked in cells treated with a Syk-selective inhibitor [18].

The receptor-mediated activation of Syk in B cells is most likely initiated by the interactions of its two, tandem, amino terminal SH2 domains with tyrosine-phosphorylated ITAMs (immunoreceptor tyrosine-based activation motifs) present on the Ig-$\alpha$ and Ig-$\beta$ components of the BCR complex [19,20]. The binding of dually phosphorylated ITAM peptides to Syk in vitro stimulates the enzyme by increasing its autophosphorylating activity [21,22] and this is likely to mirror the in vivo activation process. The consequences of these autophosphorylation events have not been explored to date, largely because no thorough study of the sites of Syk autophosphorylation has been reported. We have, therefore, undertaken to determine these sites using recombinant murine Syk, which was cloned and expressed in Sf9 cells. A combination of Edman sequencing and mass spectrometry was used to identify 10 tyrosine residues that are modified when Syk catalyzes an autophosphorylation reaction in vitro. These phosphotyrosines are localized to sites of the enzyme that are likely to modulate both its kinase activity and its interactions with SH2 domain-containing proteins.

## 2. Materials and methods

### *2.1. Materials*

A random-primed $\lambda$gt11 cDNA library derived from the murine pre-B cell line 70/Z was originally prepared by Yinon Ben-Neriah [23]. The baculovirus transfer vector pNTX was a generous gift of Harry Charbonneau (Purdue University). Restriction enzymes and DNA modifying enzymes were from Promega or New England Biolabs. An Oligolabeling Kit for DNA probe labeling was from Pharmacia Biotech. Custom oligonucleotides were synthesized on an Applied Biosystems Model 380B DNA Synthesizer at the Laboratory For Macromolecular Structure, Purdue University. A *Sequenase* (Version 2) kit from United States Biochemical was used for dideoxy DNA sequencing. [$\gamma$-$^{32}$P]ATP (> 6000 Ci/mmol) was from NEN Dupont. $\alpha$-$^{35}$S-ATP was from Amersham. Baculovirus DNA (BacPAK6) was from Clontech. Rabbit polyclonal anti-Syk peptide antibodies have been described previously [24].

### *2.2. Methods*

#### *2.2.1. Cloning and expression of murine Syk*

A full-length Syk cDNA was prepared from two overlapping clones isolated from a $\lambda$gt11, 70/Z pre-B cell, cDNA library by screening with a 515 bp murine *syk* probe [24]. An EcoR I site was engineered by site-directed mutagenesis 15 nucleotides upstream of the translational start site, using the Unique Site Elimination method [25], using the following oligonucleotide: 5′-CTTCCCAGAATTCTGAAGGGG-3′.

The Syk cDNA was inserted into the *Eco*RI/*Bam*HI site of the baculovirus expression vector pVL1392 (Invitrogen). Sf9 cells were cotransfected with pVL-Syk plasmid (2 $\mu$g) and BacPAK6 viral DNA (1 $\mu$g), and plaque purification was carried out essentially as described [26]. Recombinant plaques were amplified and screened for expression of enzymatically active Syk.

Sf9 cells were infected with high-titer virus at a multiplicity of infection of 10 for 48 h prior to harvesting. For the partial purification of Syk, cells were lysed for 10 min on ice in lysis buffer containing 1% NP-40, 10 mM Tris-HCl, pH 8.0, 1 mM $Na_3VO_4$, and a protease inhibitor cocktail of aprotinin and leupeptin (10 $\mu$g/ml each), phenylmethylsulfonylfluoride and benzamidine (1 mM each) and soybean trypsin inhibitor (100 $\mu$g/ml). The lysate was spun at $12\,000 \times g$ for 10 min at 4°C. The supernatant was applied to a column of heparin-agarose (Sigma) equilibrated in Buffer A (10 mM Hepes, pH 7.5, 10 mM NaCl, 1 mM $Na_3VO_4$ and the protease inhibitor cocktail described above). The column was washed with 20 column volumes of Buffer A followed by 10 column volumes of Buffer A containing 200 mM NaCl. Syk was eluted with 2.5 column volumes of Buffer A containing 650 mM NaCl.

*2.2.2. Cloning and expression of GSTp42.5*

A 2.4 kb *Kpn*I/*Bam*HI fragment encoding the 42.5 kDa carboxyl terminal fragment (amino acids 256–629) of Syk was subcloned into the *Kpn*I/*Bgl*II site of pNTX, generating pNTXp42.5. This vector is designed, when introduced into Sf9 cells, to direct the expression of cDNA inserts as glutathione *S*-transferase (GST) fusion proteins. pNTX possesses a *Bam*HI site just upstream of the translational start site of a sequence encoding GST. Thus, a 3.1 kb fragment encoding GSTp42.5 was subcloned into pVL1393, generating the baculovirus transfer vector pVLGSTp42.5. Sf9 cell co-transfection and plaque purification were carried out essentially as described above for full-length Syk. The cloning and expression of GST-Syk is described elsewhere [27]. GSTp42.5 and GST-Syk were isolated from the lysates of virally infected Sf9 cell lysates by adsorption to glutathione-Sepharose.

*2.2.3. Tryptic digestion of autophosphorylated kinase*

GST-fusion proteins were phosphorylated while attached to glutathione-Sepharose in buffer containing 50 mM Tris-HCl, pH 8.0, 2 mM $MnCl_2$, 1 mM $Na_3VO_4$ and 50 $\mu$M [$\gamma$-$^{32}$P]ATP (0.4 $\mu$Ci/nmol) at 30°C for 30 min. The column was washed with and resuspended in 50 mM $NH_4HCO_3$, 1 mM $Na_3VO_4$. Trypsin, at a protease to substrate ratio of 1:20 (w/w), was added and the slurry incubated overnight at 37°C with shaking. After a 16 h incubation, the column was drained and the eluate was saved. The column was washed with 400 $\mu$l aliquots of water until all radioactivity was eluted. The combined eluates were lyophilized. The resulting pellet was dissolved in water and re-lyophilized several times to remove residual $NH_4HCO_3$. In order to generate non-radiolabeled tryptic phosphopeptides, a similar phosphorylation and tryptic digestion was carried out with only unlabeled ATP in the autophosphorylation buffer.

Partially purified recombinant Syk was autophosphorylated in solution essentially as described above for the bound GST-fusion proteins, except that the specific activity of the [$\gamma$-$^{32}$P]ATP was 30.0 $\mu$Ci/nmol. Reactions were terminated by the addition of SDS-sample buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. $^{32}$P-labeled Syk was detected by autoradiography and digested on the membrane with trypsin according to [28]. Peptides released from the membrane were then lyophilized.

*2.2.4. Reverse phase HPLC analysis of tryptic digests*

Tryptic digests were solubilized in 0.1% trifluoracetic acid and separated by chromatography on a Vydac C-18 column (2.1 mm I.D. × 250 mm) using an Applied Biosystems 172A gradient HPLC system. Peptides were eluted with a linear gradient from 0% to 30% acetonitrile in 0.1% TFA in 63 min followed by a linear gradient to 70% acetonitrile in 0.1% TFA in 10 min at a flow rate of 150 $\mu$l/min. Peptides were monitored by absorbance at 214 nm and phosphopeptides by liquid scintillation spectrometry. Where necessary, phosphopeptides were rechromatographed using an Applied Biosystems phenyl column (2.1 mm I.D. × 250 mm) using the gradient described above or a linear gradient from 0% to 60% acetonitrile in 60 min at 200 $\mu$l/min.

*2.2.5. Mass spectrometry of phosphopeptides*

Analysis of tryptic peptides by mass spectrometry was performed using a Vestec (Perseptive Biosystems) matrix-assisted laser desorption time-of-flight (MALDI-TOF) instrument, made available through the generosity of Dr. Raymond D. Kaiser at Eli Lilly and Company (Indianapolis, IN). Biosynthetic Human Insulin (BHI, $MH^+ = 5809.7$) was used as an internal standard for calibration. All spectra were collected using 4-hydroxy $\alpha$-cyano-cinnamic acid as the matrix. Average masses were calculated using MacPromass software, a gift from Dr. Terry D. Lee (Beckman Research Institute at the City of Hope, Duarte, CA).

*2.2.6. Polyacrylamide gel electrophoresis of tryptic digests*

Electrophoretic separation of phosphopeptides was carried out essentially as described [29]. Briefly, tryptic digests of $^{32}$P-labeled Syk or GST-fusion proteins were resolved on alkaline 40% polyacrylamide gels, which were then dried onto Whatman 3MM chromatography paper. Phosphopeptides were detected by autoradiography.

*2.2.7. PLC-$\gamma$1 SH2 binding studies*

*E. coli* harboring expression plasmids encoding GST fusion proteins of bovine PLC-$\gamma$1 amino and carboxyl terminal SH2 domains (generous gift of Tony Pawson, Samuel Lunenfeld Institute, Toronto) were grown overnight in 2X YT medium, diluted 3-fold in fresh medium and induced with isopropylthiogalactoside (200 $\mu$M) for 3 h. Cells were lysed and GST fusion proteins were isolated on glutathione-Sepharose beads as described previously [30]. Recombinant Syk was autophosphorylated in a 250 $\mu$l reaction essentially as described above, except that BSA (100 $\mu$g/ml) was included in the reaction. The reaction was terminated on ice with 500 mM EDTA (5 $\mu$l) and 100 $\mu$l aliquots of the reaction were added to 400 $\mu$l suspensions of bead-immobilized GST SH2 fusion proteins ($\sim$ 10 $\mu$g protein in 400 $\mu$l of PBS containing 1% Triton X-100). The slurries were rotated at $4^0$C for 1 h. After aspiration of the supernatant, the beads were washed 3 times with PBS/1% Triton (1 ml), 3 times with PBS, and boiled in SDS-sample buffer (150 $\mu$l). Bound proteins were separated by SDS/PAGE and detected by autoradiography.

## 3. Results

### *3.1. Molecular cloning of murine syk cDNA*

RT-PCR was carried out on total RNA from the murine B cell line L10.A to generate a 515 bp probe [24], which was used to screen a cDNA library derived from the murine pre-B cell line 70/Z. Three independent clones (55, 28B and LLA) were isolated. The library inserts, all of which were approx. 3 kb in length, were subcloned into pBluescript KS 11 and sequenced. None of the clones contained the complete coding region of murine *syk*. Clones 55 and 28B were truncated at the 5′-end at codons for Asp 68 and Ala 82, respectively. Clone LLA was truncated at the 3′ end at the codon for Asp 606. This clone was presumably derived from partially spliced heteronuclear RNA since the sequence immediately downstream from the truncation point was intronic (Victor Tybulewicz, MRC London, personal communication). Full-length murine *syk* cDNA, deduced from the three isolated clones, encodes a 629 amino acid protein with a calculated molecular mass of 71 330 Da (Fig. 1) (Genbank Accession # U25685). Murine Syk shares $>$ 90% amino acid sequence identity with the previously reported porcine, rat and human Syk sequences [15,31,32].

### *3.2. Identification of multiple sites of autophosphorylation on GST-Syk by HPLC*

The engagement of antigen receptors on B cells leads to a rapid increase in the tyrosine-phosphorylation of Syk, an event that coincides with changes in the catalytic activity of the kinase [11–15] and its interactions with downstream signaling molecules [33,34]. Many of these changes occur in the absence of exogenous tyrosine kinases, indicating that the autophosphorylating activity of Syk is critical to its function [35]. We set out, therefore, to identify those tyrosine residues on Syk that are covalently modified following its incubation with ATP.

To obtain sufficient, purified enzyme for this anal-

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                 [-------  ----------  --  SH2  N  -  ----------
1   MAGSAVDSAN  HLTYFFGNIT  REEAEDYLVQ  GGMTDGLYLL  RQSRNYLGGF
    ----------  ----------  ----------  ----------  ----------
51  ALSVAHNRKA  HHYTIERELN  GTYAISGGRA  HASPADLCHY  HSQEPDGLIC
    ------]
101 LLKKPFNRPP  GVQPKTGPFE  DLKENLIREY  VKQTWNLQGQ  ALEQAIISQK
                   [----  ----------  --  SH2  C  -  ----------
151 PQLEKLIATT  AHEKMPWFHG  NISRDESEQT  VLIGSKTNGK  FLIRARDNSG
    ----------  ----------  ----------  ----------  ----------
201 SYALCLLHEG  KVLHYRIDRD  KTGKLSIPEG  KKFDTLWQLV  EHYSYKPDGL
    --------]
251 LRVLTVPCQK  IGAQMGHPGS  PNAHPVTWSP  GGIISRIKSY  SFPKPGHKKP

301 APPQGSRPES  TVSFNPYEPT  GGPWGPDRGL  QREALPMDTE  VYESPYADPE
                 [-------  --KINASE--  ----------  ----------
351 EIRPKEVYLD  RSLLTLEDNE  LGSGNFGTVK  KGYYQMKKVV  KTVAVKILKN
    ----------  ----------  ----------  ----------  ----------
401 EANDPALKDE  LLAEANVMQQ  LDNPYIVRMI  GICEAESWML  VMEMAELGPL
    ----------  ----------  ----------  ----------  ----------
451 NKYLQQNRHI  KDKNIIELVH  QVSMGMKYLE  ESNFVHRDLA  ARNVLLVTQH
    ----------  ----------  ----------  ----------  ----------
501 YAKISDFGLS  KALRADENYY  KAQTHGKWPV  KWYAPECINY  YKFSSKSDVW
    ----------  ----------  ----------  ----------  ----------
551 SFGVLMWEAF  SYGQKPYRGM  KGSEVTAMLE  KGERMGCPAG  CPREMYDLMN
    ----------  ----------  ]
601 LCWTYDVENR  PGFTAVELRL  RNYYYDVVN/
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Fig. 1. Amino acid sequence of murine Syk. The amino acid sequence of full-length murine Syk was deduced from clones 55 and LLA, which were isolated and sequenced as described in Section 2. The bounderies of the two SH2 domains and the kinase domain are indicated by brackets.

ysis, Syk was expressed in Sf9 cells as a fusion protein coupled to GST (GST-Syk). A full-length Syk cDNA was constructed from two of the partial clones (55 and LLA) and inserted into a vector containing the coding sequence for GST. GST-Syk, after partial purification by immobilization on glutathione-Sepharose beads, efficiently catalyzed an autophosphorylation reaction when incubated with [$\gamma$-$^{32}$P]ATP (Fig. 2).

To begin an analysis of the sites modified during autophosphorylation, purified, autophosphorylated GST-Syk was extensively digested with trypsin. Tryptic peptides were separated by reverse phase HPLC on a C-18 column. Peptides were collected by O.D.$_{214}$ and crude phosphopeptides were identified by scintillation counting. Analysis of the resulting profile, shown in Fig. 3A, indicated that the autophosphorylation of GST-Syk in vitro led to the covalent modification of numerous sites.

### 3.3. Autophosphorylation of GSTp42.5

The amino terminal half of Syk contains two SH2 domains that govern the interactions of the kinase with ITAMs on antigen receptors while the carboxyl terminal half contains the catalytic domain. These functional domains are separated by a 'hinge' region that is highly susceptible to proteolysis [31,36]. To identify the domain(s) containing the major sites of autophosphorylation, a 42.5 kDa carboxyl terminal fragment of murine Syk, which lacks the tandem SH2 domains, was expressed in Sf9 cells as a GST-fusion protein (GSTp42.5). As shown in Fig. 2, GSTp42.5, isolated from insect cell lysates by adsorption to glutathione-Sepharose, was also active as a kinase and readily catalyzed an autophosphorylation reaction when incubated with [$\gamma$-$^{32}$P]ATP.

GSTp42.5 was autophosphorylated and digested with trypsin in an identical manner to GST-Syk. The HPLC (C-18) profile of the resulting phosphopeptides was essentially identical to that of GST-Syk (Fig. 3B) except for the absence of peptide 1 (Fig. 3A). Also in this chromatogram, peak 6 was clearly resolved into three separate peaks.

### 3.4. Identification of phosphopeptides derived from GSTp42.5 and GST-Syk

Electrophoresis of a small aliquot of Peak 6 on a 40% polyacrylamide alkaline gel [29] followed by

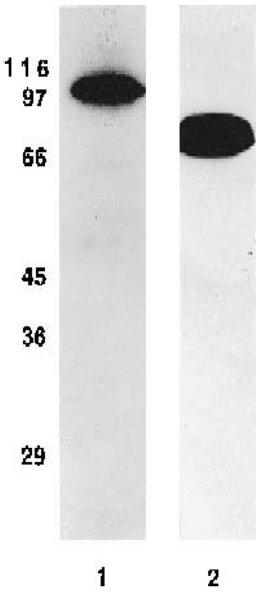


Fig. 2. In vitro autophosphorylation of recombinant GST-kinase fusion proteins. GST-Syk (lane 1) and GSTp42.5 (lane 2), isolated from Sf9 cells by adsorption to glutathione-Sepharose, were autophosphorylated in the presence of [$\gamma$-$^{32}$P]ATP, separated by SDS-PAGE and detected by autoradiography. The migration positions of mol. wt. standards are indicated in kDa.

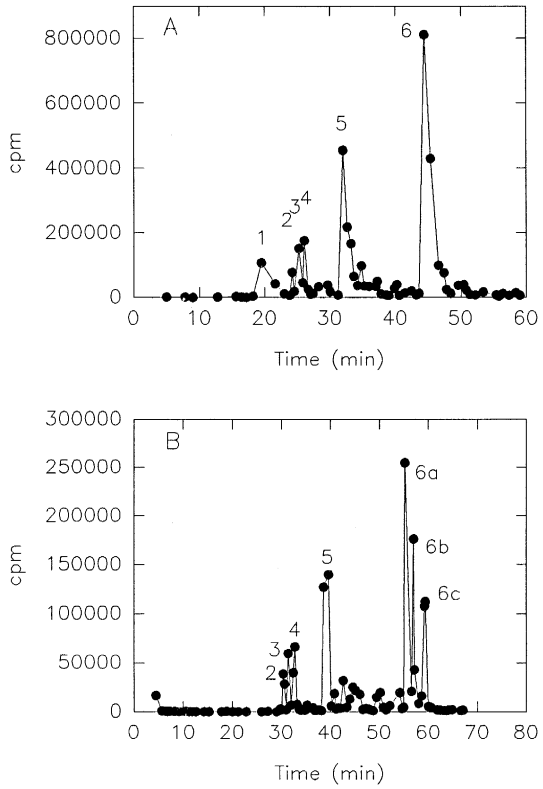


Fig. 3. Separation of tryptic phosphopeptides from in vitro autophosphorylated GST-Syk and GSTp42.5. GST-Syk (A) or GSTp42.5 (B) were autophosphorylated in the presence of [$\gamma$-$^{32}$P]ATP and digested with trypsin as described in Section 2. Tryptic peptides were then separated by reverse phase HPLC on a C-18 column. Peptides were collected by absorbance at 214 nm and phosphopeptides were detected by Cherenkov counting.

autoradiography of the separated peptides (Fig. 4A) confirmed the presence of three distinct phosphopeptides (6a, 6b and 6c). Crude GSTp42.5 tryptic phosphopeptides corresponding to peaks 6a, 6b and 6c derived from the C-18 column were further purified on a phenyl column (Fig. 5). Each peak contained a single, major phosphopeptide. These were collected and subjected to Edman sequence analysis. Partial sequence analysis of peaks 6a and 6c gave identical results (Table 1), even though they were well separated on HPLC. This anomaly was resolved by subjecting these two peptides to MALDI-TOF mass spectrometry (Fig. 6). The observed ions for peptides 6a and 6c ($MH^+$ = 2842.08 and 2760.94 amu, respectively), were in close agreement with the predicted molecular masses of the di- and monophosphorylated forms of the hinge region-derived tryptic peptide encompassing amino acids 333–355 (calculated $MH^+$ = 2840.95 and 2760.95 amu, respectively). In order to determine which tyrosine residue(s) in the monophosphopeptide was modified, peptide 6c was re-subjected to Edman sequencing (20 cycles). Tyrosine was observed at cycle 10, but not at cycle 14, suggesting that this peptide was phosphorylated exclusively on Tyr 346.

Partial sequence analysis (10 cycles) of peptide 6b indicated that this was another hinge region-derived tryptic peptide, corresponding to amino acids 299–328 (Table 1). Mass spectrometric analysis (Fig. 6) produced a molecular ion ($MH^+$ = 3289.95), that was consistent with the phosphorylated (Tyr 317) peptide (calculated $MH^+$ = 3290.49).

GSTp42.5 phosphopeptides corresponding to peak 5 were further purified by phenyl column HPLC (Fig. 7). Peak 5 generated 3 major phosphopeptide-containing peaks. Analysis of each peak by 40% polyacrylamide alkaline gel electrophoresis indicated that peaks eluting at 17 and 21.5 min each contained a single phosphopeptide (5a and 5b, respectively) while the peak eluting at 22.5 min contained two different phosphopeptides (5c and 5d) (Fig. 4B). Edman se-

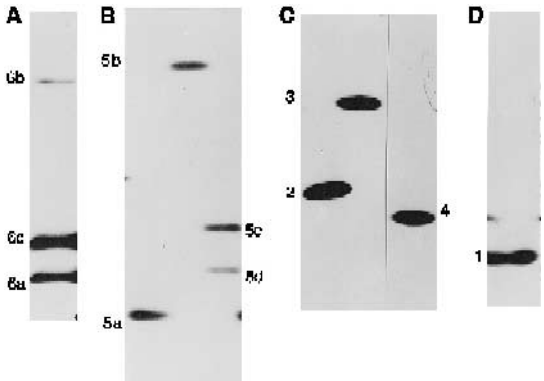


Fig. 4. Separation of phosphopeptides by electrophoresis on 40% polyacrylamide alkaline gels. Aliquots of peaks derived from the HPLC separation of various phosphopeptides were evaporated to dryness, dissolved in sample buffer and separated on 40% alkaline gels as described in Section 2. Phosphopeptides were detected by autoradiography. Peptides 6a–6c (A) were derived from peak 6, Fig. 3A, and are labeled in the order in which they elute from a phenyl HPLC column. Peptides 5a–5d (B) are derived from phenyl column HPLC separation of peak 5 (Fig. 7) and are labeled in the order in which they eluted. Peptides 2–4 (C) and peptide 1 (D) are derived from the corresponding peaks (Fig. 3A).

Table 1
Sequences of tryptic phosphopeptides derived from autophosphorylated Syk

| Peptide [a] | Residues | Phosphotyrosine | Sequence [b] |
|---|---|---|---|
| 1 | 129–132 | 130 | EpYVK |
| 2 | 515–521 | 519/520 | ADENpYpYK |
| 3 | 515–527 | 519/520 | ADENpYpYKAQTHGK |
| 4a | 515–521 | 520 | ADENYpYK |
| 4b | 515–521 | 519 | ADENpYYK |
| 5a | 356–361 | 358 | EVpYLDR |
| 5b | 289–298 | 290 | SpYSFPKPGHK |
| 5c | 620–629 | 623/624/625 | LRNYpYpYDVVN |
| 5d | 622–629 | 623/624/625 | NYpYpYDVVN |
| 6a | 333–355 | 342/346 | EALPMDTEVpYESPpYADPEEIRPK |
| 6b | 299–328 | 317 | KPAPPQGSRPESTVSFNPpYEPTGGPWGPDR |
| 6c | 333–355 | 346 | EALPMDTEVYESPpYADPEEIRPK |

[a] Peptides were purified by phenyl column HPLC of the C-18 column HPLC peaks 1-6 (Fig. 3) and characterized as described in Section 2.
[b] Phosphorylated tyrosine residues are denoted as pY.

quence analyses indicated that peptides 5a and 5b corresponded to amino acids 356–361 and 289–298, respectively (Table 1). The absence of tyrosine at the appropriate sequencing cycles confirmed that Tyr 358 and Tyr 290, respectively, were phosphorylated in these two peptides. Sequence analysis of the peak eluting at 22.5 min confirmed the presence of two phosphopeptides. These phosphopeptides, 5c and 5d, were related by incomplete proteolysis, and corresponded to amino acids 620–629 and 622–629, respectively. Mass spectrometric analysis of peptide 5c produced a molecular ion ($MH^+ = 1480.3$) that was consistent with that of a doubly phosphorylated peptide (calculated $MH^+ = 1479.5$) (Fig. 8A). Edman sequencing analysis of peptide 5d yielded tyrosine only in cycle two, suggesting that Tyr 624 and 625 were the principal sites of phosphorylation.

Peaks 2, 3 and 4 from GSTp42.5 all corresponded to peptides that contained tyrosines 519/520 (Table 1). Peaks 2 and 3 each contained a single phosphopeptide as analyzed by 40% polyacrylamide alkaline gel electrophoresis (Fig. 4C) and by phenyl column HPLC (Fig. 9A and B). As determined by Edman sequencing, these peptides corresponded to amino acids 515–521 and 515–527, respectively, with peptide 3 corresponding to an incompletely proteolyzed form of peptide 2. These peptides were phosphorylated on both tyrosine residues, as evidenced by the absence of detectable tyrosine at cycles 5 and 6 in both sequences. Mass spectrometric analysis of pep-

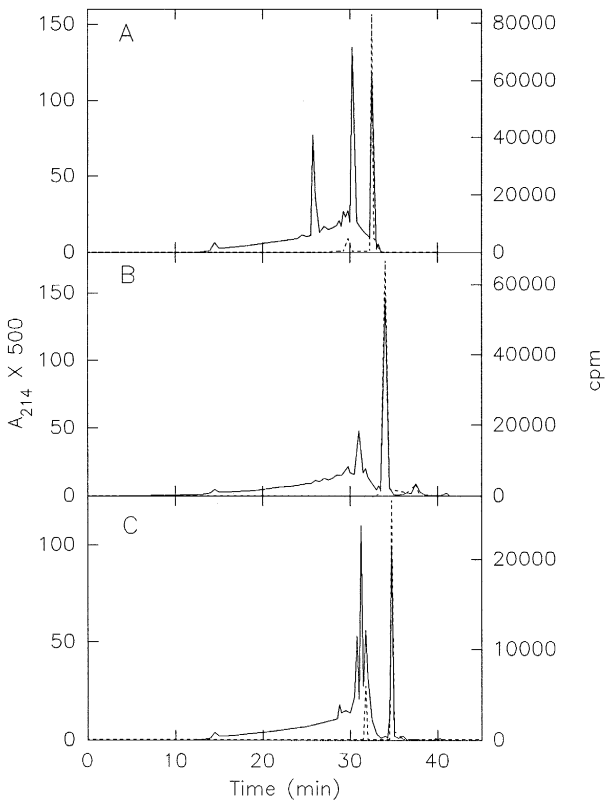


Fig. 5. Separation of phosphopeptides 6a, 6b and 6c by phenyl column HPLC. Peak fractions corresponding to peaks 6a (A), 6b (B) and 6c (C) of Fig. 3B were separately chromatographed on a phenyl column. Peptides were detected by absorbance at 214 nm (solid line) and by scintillation spectrometry (dashed line).

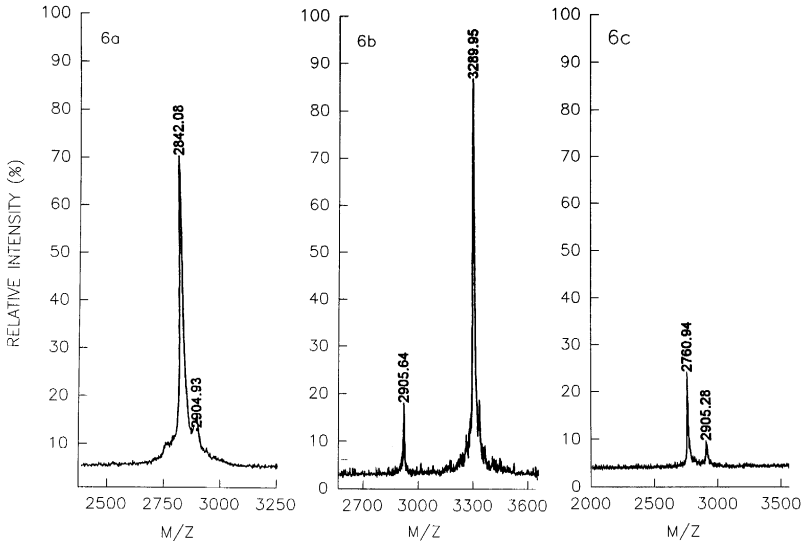


Fig. 6. Mass spectra of peptides 6a, 6b and 6c. Peptides 6a, 6b and 6c (shown in Fig. 5) were characterized by mass spectrometry as described in Section 2. The minor peaks near 2905 (M/Z) correspond to the doubly protonated internal standard (BHI).

tide 3 yielded a molecular ion ($MH^+ = 1686.7$) consistent with that of the doubly phosphorylated peptide (calculated $MH^+ = 1685.7$) (Fig. 8B).

Peak 4 yielded a single phosphopeptide by 40% polyacrylamide alkaline gel electrophoresis (Fig. 4C), but gave rise to two phosphopeptides (4a and 4b) following chromatography on the phenyl column (Fig. 9C and D). By Edman sequencing, these peptides were identified as monophosphorylated forms of peptide 2, and were distinguishable by the absence of detectable tyrosine at either cycle 6 or 5 of their respective sequences.

As mentioned above, tryptic digestion of GST-Syk produced an essentially identical HPLC (C-18 column) profile to that derived from GSTp42.5, with the exception of an additional, early eluting phosphopeptide (peptide 1 in Fig. 3A). Analysis by 40% polyacrylamide alkaline gel electrophoresis indicated the presence of a single phosphopeptide in peak 1 (Fig.

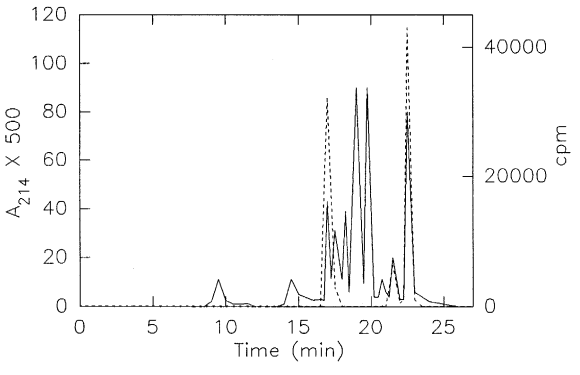


Fig. 7. Separation of peak 5 phosphopeptides by phenyl column HPLC. Peak fractions corresponding to peak 5 of Fig. 3B were chromatographed on a phenyl column. Peptides were detected by absorbance at 214 nm (solid line) and by scintillation spectrometry (dashed line).

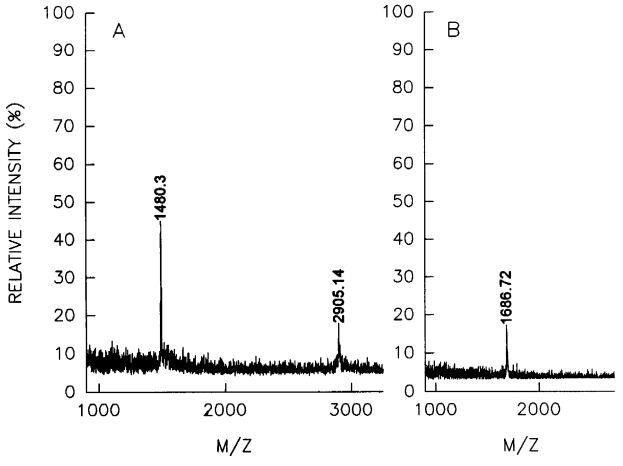


Fig. 8. Mass spectra of peptides 5c and 3. Peptides 5c (Fig. 7) (A) and 3 (Fig. 9) (B) were characterized by mass spectrometry as described in Section 2.

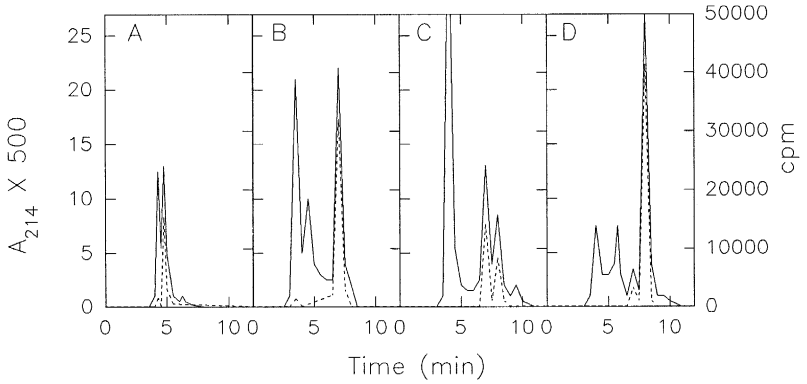


Fig. 9. Separation of phosphopeptides 2, 3 and 4 by phenyl column HPLC. Peak fractions corresponding to peaks 2 (A), 3 (B) and the left- (C) and right-hand (D) sides of peak 4 (C and D) of Fig. 3B were separately chromatographed on a phenyl column. Peptides were detected by absorbance at 214 nm (solid line) and by scintillation spectrometry (dashed line).

4D). This peptide was purified by HPLC on a phenyl column (data not shown) and identified by Edman sequencing as corresponding to amino acids 129–132, which lie between the two SH2 domains of Syk. The absence of detectable tyrosine at cycle 2 was consistent with the presence of phosphotyrosine at residue 130. This phosphopeptide was missing from tryptic digests of an autophosphorylated, mutant form of Syk in which Tyr 130 had been mutated to Phe (Keshvara, L. and Geahlen, R.L. unpublished data).

### *3.5. Autophosphorylation of recombinant Syk*

To determine which of the autophosphorylation sites from the GST-Syk were present in autophosphorylated Syk, partially purified recombinant Syk was phosphorylated, separated by SDS-PAGE and transferred to nitrocellulose. Syk was the only phosphorylated protein detected by autoradiography (Fig. 10A). Tryptic digestion of Syk off of the membrane produced a series of peptides whose C-18 HPLC profile was similar to that of GST-Syk (data not shown). Syk-derived radiolabeled peptides eluting at similar times to those derived from the GST fusion proteins were compared to the latter by separation on 40% alkaline gel electrophoresis followed by autoradiography. A representative autoradiogram, demonstrating the presence of peptides 6a, 6b and 6c in autophosphorylated Syk, is shown in Fig. 10B. These comparisons demonstrated that all 10 of the phosphotyrosine residues present in autophosphorylated GST-Syk were present in the recombinant enzyme. Thus, the presence of the GST moiety on GST-Syk did not generate any new autophosphorylation sites not found on the wild-type enzyme.

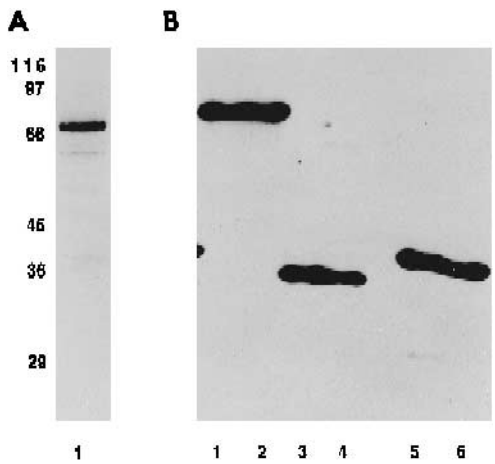


Fig. 10. Sites of autophosphorylation in recombinant Syk. (A) In vitro autophosphorylation of recombinant Syk. Partially purified recombinant Syk was autophosphorylated in the presence of [$\gamma$-$^{32}$P]ATP as described in Section 2. Proteins were separated by SDS-PAGE and phosphorylated Syk was detected by autoradiography. The migration positions of molecular weight standards are indicated in kDa. (B) 40% polyacrylamide alkaline gel comparison of peptides 6a, 6b and 6c from GSTp42.5 and recombinant Syk. HPLC-purified phosphopeptides 6b, 6a and 6c, generated by tryptic digestion of autophosphorylated GSTp42.5 (lanes 1, 3 and 5) and Syk (lanes 2, 4 and 6) were separated by 40% polyacrylamide alkaline gel electrophoresis as described in Section 2. Peptides were detected by autoradiography.

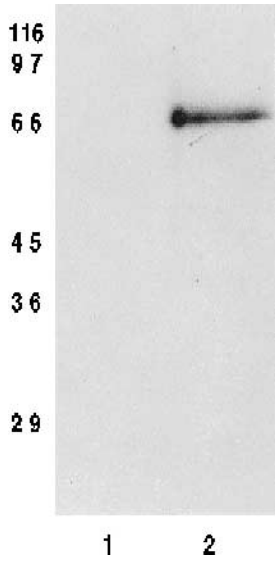


Fig. 11. Interaction of Autophosphorylated recombinant Syk with SH2 domains of PLC-$\gamma$1. Recombinant Syk was autophosphorylated in the presence of [$\gamma$-$^{32}$P]ATP and incubated with glutathione-Sepharose-immobilized GST fusion proteins of either the amino (lane 1) or carboxyl (lane 2) terminal SH2 domains of bovine PLC-$\gamma$1 as described in Section 2. After washing of the beads, bound Syk was separated by SDS-PAGE and detected by autoradiography.

### *3.6. Analysis of interaction between autophosphorylated recombinant Syk and SH2 domains of PLC-$\gamma$1*

Phosphorylated tyrosine residues often mediate protein binding to SH2 domains of other proteins [8]. Three of the Syk autophosphorylation sites determined above (Tyr 290, 342 and 346) are each part of a ($PO_4$)Tyr X X Pro motif, which suggests that they may form binding sites for the carboxyl terminal SH2 domain of PLC-$\gamma$1 [38]. Given the recent report of the association with Syk with PLC-$\gamma$1 in lysates of activated B cells [33], we investigated the in vitro association of autophosphorylated recombinant Syk with each SH2 domain of this signaling protein. Syk was autophosphorylated in the presence of [$\gamma$-$^{32}$P]ATP and incubated with immobilized GST fusion proteins of PLC-$\gamma$1 amino and carboxyl terminal SH2 domains. Syk-PLC-$\gamma$1 SH2 domain interactions were detected by autoradiography (Fig. 11). Radiolabeled Syk bound preferentially to the PLC-$\gamma$1 carboxyl terminal SH2 fusion protein.

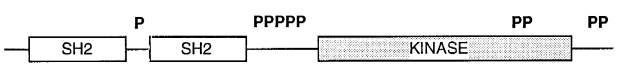


Fig. 12. Relative locations of autophosphorylation sites in murine Syk.

## 4. Discussion

The Syk protein-tyrosine kinase becomes tyrosine phosphorylated and activated in response to the aggregation of immune recognition receptors on a variety of hematopoietic cell types. The clustering of these antigen receptors initiates a cascade of biochemical events beginning with the increased phosphorylation of proteins such as PLC-$\gamma$ on tyrosine. These, in turn, couple receptor engagement to increases in the levels of intracellular second messengers such as calcium and inositol 1,4,5-trisphosphate ($IP_3$). These responses are generally blocked in cells in which Syk has been either deleted or inhibited, indicating that Syk is a critical component of this signaling machinery [16–18]. The binding of Syk to clustered, phosphorylated ITAMs induces its phosphorylation and activation [39]. The kinase or kinases that catalyze the phosphorylation of Syk likely include Syk itself as well as members of the Src-family of protein-tyrosine kinases [40]. In mast cells and T cells, clustering of a chimeric receptor containing the extracellular domain of CD16 and Syk as a cytoplasmic domain is sufficient to reproduce the full spectrum of signals generated by aggregation of each respective receptor (i.e., T cell antigen receptor or Fc$\epsilon$RI) [35,41]. It is likely, therefore, that Syk itself is capable of catalyzing the bulk of the phosphorylation reactions that are important to its activation and subsequent involvement in cell signaling pathways. To begin to understand how the autophosphorylation of Syk might be involved in signaling, we initiated a study to map the major sites of autophosphorylation.

Our analysis of autophosphorylation sites indicate that at least ten different tyrosine residues can be covalently modified following the incubation of Syk with [$\gamma$-$^{32}$P]ATP (Table 1). The relative locations of these sites are indicated on the model shown in Fig. 12. The first site, beginning from the amino terminus, is Tyr-130. Tyr-130 is present in a short tryptic peptide, EYVK, that is located between the two

tandem SH2 domains that make up the amino terminal half of Syk. Tyr-130 and the amino acids surrounding it are highly conserved in Syk cDNAs cloned from murine, rat, porcine and human species (Fig. 1; [15,31,32,42]), suggesting that this region may play an important functional role. Tyr-130 is also analogous to Tyr-126 of the Syk-related kinase, ZAP-70, which is found within the related sequence, DYVR [43]. Tyr-126 has previously been identified as a site of autophosphorylation in recombinant ZAP-70 [44]. The consequences of phosphorylation at Tyr-130 are uncertain, but its presence between the tandem SH2 domains suggests that it could play a role in modulating the interactions of Syk with the ITAMs present on receptor components. Following receptor engagement, Syk associates with phosphorylated ITAMs on clustered receptors, but is released following tyrosine phosphorylation and activation [27]. The phosphorylation of Tyr-130 could conceivably play a role in the release of the activated kinase. Alternatively, phosphorylation at this site may modulate the interactions of Syk or ZAP-70 with other proteins.

Five sites of autophosphorylation lie within a hinge or spacer region that separates the SH2 domains from the carboxyl terminal catalytic domain. In Syk, this hinge is likely to be exposed since the enzyme is very sensitive to proteolytic cleavage within this region [31,36]. Proteolysis yields an active carboxyl terminal, catalytic fragment of approximately 40 kDa; and it was this 40 kDa form of Syk that was first isolated from bovine thymus and porcine spleen [45,46]. The first site in the hinge region, Tyr-290, is present in a 23 amino acid insert that is missing from ZAP-70 and from a shorter, alternatively spliced variant of Syk [42]. It is, however, highly conserved in the most abundant form of Syk from all species that have been examined. The second site, Tyr-317, is also conserved in all Syk species, but amino acid residues distal to this site are somewhat variable. This tyrosine is localized to a region analogous to that of Tyr-292, an autophosphorylation site identified in the spacer region of ZAP-70 [44]. The sequence surrounding Tyr-317 (PYEPT) is also similar to that found in ZAP-70 (YTPEP).

The peptide containing the third and fourth sites, Tyr-342 and Tyr-346, was isolated in both mono- and di-phosphorylated forms (Table 1 - peptides 6c and 6a, respectively). The monophosphorylated peptide (6c) contained phosphotyrosine only at position 346, suggesting that phosphorylation of Tyr-342 and Tyr-346 occurs in an ordered fashion. The amino acids surrounding these tyrosines are completely conserved in all forms of Syk and are highly similar to an analogous region found in ZAP-70. In general, the spacer regions of Syk and ZAP-70 show little sequence similarity with the exception of a 16 amino acid stretch that surrounds these tyrosines [42]. These analogous tyrosines have not been identified as sites of autophosphorylation in ZAP-70 [44]. They are, however, rapidly phosphorylated in Syk, even with low concentrations of ATP and short incubation times (unpublished observations). The fifth site, Tyr-358, is completely conserved among all Syk sequences reported to date, but lies within a region that bears no sequence similarity to ZAP-70 [42].

Since the five sites of autophosphorylation in the spacer lie within the exposed hinge region, it is attractive to speculate that they mediate protein-protein interactions. Ligand-induced autophosphorylation of receptor PTKs generates phosphotyrosine-based motifs that bind SH2 domains of various downstream signaling proteins, including PLC-$\gamma$, PI3-kinase and many others [8]. Syk and ZAP-70 may act in a similar fashion. ZAP-70, when bound to phosphorylated ITAMs, becomes phosphorylated on multiple tyrosines and gains the ability to bind the SH2 domains of Fyn, Lck, Abl and rasGAP [47]. ZAP-70 has also been reported to interact directly with Vav in an interaction mediated by the Vav SH2 domain [48]. Syk, in turn, has been shown to physically associate with PLC-$\gamma$1 [33] and with the Src-family kinases Lyn and Blk through their SH2 domains [34]. Using a phosphopeptide library affinity selection technique, Cantley and co-workers have determined that the affinity of a given SH2 domain for an interacting protein is governed principally by the identity of the three amino acids that reside immediately carboxyl terminal to the interacting phosphotyrosine residue of that protein [38,49]. For example, these studies predict that the amino and carboxyl terminal SH2 domains of PLC-$\gamma$1 should bind preferentially to $(PO_4)$Tyr-Leu-Glu/Asp-Leu and $(PO_4)$Tyr-Val/Ile-X-Pro, respectively [38]. The predicted binding preference of the carboxyl terminal SH2 domain of PLC-$\gamma$1 corresponds most closely to three phospho-

tyrosine-containing motifs present in the hinge region of autophosphorylated Syk: ($PO_4$)Tyr-290-Ser-Phe-Pro, ($PO_4$)Tyr-342-Glu-Ser-Pro and ($PO_4$)Tyr-346-Ala-Asp-Pro. This specificity is consistent with the observation that the carboxyl terminal, but not the amino terminal SH2 domain of PLC-$\gamma$1 efficiently binds autophosphorylated Syk in vitro (Fig. 11). Within the cell, such an interaction could facilitate the binding and tyrosine phosphorylation of PLC-$\gamma$1 by Syk. The tyrosine phosphorylation of PLC-$\gamma$1 on Tyr 783 and Tyr 1254 has been implicated in its activation in B cells, T cells and fibroblasts [50–52], and Syk has been shown to phosphorylate peptides that correspond to regions of PLC-$\gamma$1 known to be phosphorylated in lymphocytes [53]. In fact, a recent study indicates that PLC-$\gamma$1 does, indeed, interact with phosphotyrosine residues present within the hinge region and serves as a substrate for Syk [54].

Two sites of Syk autophosphorylation (Tyr-519 and Tyr-520) are located within the catalytic domain. These residues had been identified as autophosphorylation sites in a previous report [37]. Analogous sites of autophosphorylation are found in activation loops present in the catalytic domains of most protein-tyrosine kinases and their phosphorylation plays a positive role in activation. As shown for Tyr-1162 of the insulin receptor, the unphosphorylated tyrosine is bound in the active site where it precludes the binding of ATP [55]. The phosphorylation of Tyr-1162 then relieves this inhibition and the kinase is now active. In ZAP-70, Tyr-493 appears to be most analogous to the insulin receptor Tyr-1162. A Tyr-493 to Phe-493 mutant of ZAP-70 exhibits low basal activity and is no longer activated by tyrosine phosphorylation [5]. Interestingly, an adjacent residue, Tyr-492, when mutated to Phe, results in a constitutively active form of ZAP-70 [5]. Its exact role in regulating the activity of the kinase has yet to be determined.

Tyrosines 519 and 520 of Syk are analogous to tyrosines 492 and 493 of ZAP-70. Both singly and dually phosphorylated forms of the peptide containing residues 519 and 520 were isolated. The presence of two phosphates on the peptide inhibited proteolysis at Lys-521 leading to the recovery of a phosphopeptide that was six amino acids longer than the expected one. Singly phosphorylated forms of the peptide were efficiently cleaved at Lys-521 and both of the possible monophosphorylated peptides were recovered. This suggests that the phosphorylation of these tyrosines are not likely to occur in a specific order.

It is interesting to note that ZAP-70 does not catalyze the autophosphorylation of tyrosines 492 and 493, but that these sites can be phosphorylated by Src-family kinases such as Lck [44]. In JCam.1 T-cells, which lack active Lck, engagement of the T cell antigen receptor fails to activate ZAP-70 and fails to generate a biochemical signal, indicating that phosphorylation by Lck is a likely prerequisite for activation [56]. The ability of Syk, but not ZAP-70, to catalyze these autophosphorylations may explain some of the functional differences between these kinases. For example, while the CD16/Syk chimeric receptors described above can signal effectively in transfected T cells [35], the corresponding CD16/ZAP-70 chimeras do not, unless they are co-crosslinked with chimeras bearing a Src-family kinase such as Fyn as a cytoplasmic domain. Thus, ZAP-70 is dependent on a Src-family kinase for phosphorylation of the tyrosines present on the activating loop while Syk appears to have the capacity to autoactivate.

The final two sites, tyrosines 624 and 625 are located in the extreme carboxyl terminus of Syk. Tyrosines 623, 624 and 625 are completely conserved in all forms of Syk. These three tyrosines are also found in murine ZAP-70; two of the three are found in the human homolog. ZAP-70 has a 17 amino acid carboxyl terminal extension that is lacking in Syk. In all Src-family kinases, a tyrosine located near the carboxyl terminus plays an important role in regulating enzyme activity [1]. Phosphorylation of this site by the Csk protein-tyrosine kinase negatively regulates the activity of the Src-kinases [57]. It is not yet known if the carboxyl terminal tyrosines play a similar role in regulating the activities of the Syk-family enzymes. An antibody directed against the carboxyl terminus of Syk can block its activation in solution by phosphorylated ITAM peptides, suggesting that this region of the molecule may, in fact, play a role in enzyme regulation [21]. This role could conceivably involve the phosphorylation of one or more tyrosines present in this region.

In summary, we have determined the sites of in vitro autophosphorylation of the hematopoietic PTK

Syk and provided evidence for a docking function for at least one of these sites. In order to ascribe physiological significance to any of these in vitro sites, their occurrence in unactivated and/or activated hematopoietic cell types must first be demonstrated. The specific functional significance of each site must also await studies on site-directed mutants of Syk. Efforts are underway to determine the in vivo sites of Syk phosphorylation and their roles in signal transduction.

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## References

[1] Piwnica-Worms, H., Saunders, K.B., Roberts, T.M., Smith, A.E. and Cheng, S.H. (1987) Cell 49, 75–82.
[2] Kmiecik, T.E. and Shalloway, D. (1987) Cell 49, 65–73.
[3] Abraham, N. and Veillette, A. (1990) Mol. Cell. Biol. 10, 5197–5206.
[4] Zhang, B., Tavares, J.M., Leland, E. and Roth, R.A. (1991) J. Biol. Chem. 266, 990–996.
[5] Wange, R.L., Guitian, R., Isakov, N., Watts, J.D., Aebersold, R. and Samelson, L.E. (1995) J. Biol. Chem. 270, 18730–18733.
[6] Ullrich, A. and Schlessinger, J. (1990) Cell 61, 203–212.
[7] der Geer, P., Hunter, T. and Lindberg, R.A. (1994) Annu. Rev. Cell Biol. 10, 251–337.
[8] Pawson, T. (1995) Nature 373, 573–580.
[9] Claesson-Welsh, L. (1994) J. Biol. Chem. 269, 32023–32026.
[10] Defranco, A.L. (1994) Curr. Opin. Immunol. 6, 364–371.
[11] Hutchcroft, J.E., Harrison, M.L. and Geahlen, R.L. (1992) J. Biol. Chem. 267, 8613–8619.
[12] Leprince, C., Draves, K.E., Geahlen, R.L., Ledbetter, J.A. and Clarke, E.A. (1993) Proc. Natl. Acad. Sci. USA 90, 3236–3240.
[13] Yamada, T., Taniguchi, T., Yang, C., Yasue, S., Saito, H. and Yamamura, H. (1993) Eur. J. Biochem. 213, 455–459.
[14] Hutchcroft, J.E., Harrison, M.L. and Geahlen, R.L. (1991) J. Biol. Chem. 266, 14846–14849.
[15] Law, C.L., Sidorenko, S.P., Chandran, K.A., Draves, K.E., Chan, A.C., Weiss, A., Edelhoff, S., Disteche, C.M. and Clark, E.A. (1994) J. Biol. Chem. 269, 12310–12319.
[16] Turner, M., Mee, J., Costello, P.S., Williams, O., Price, A.A., Duddy, L.P., Furlong, M.T., Geahlen, R.L. and Tybulewicz, V.L.J. (1995) Nature, in press.
[17] Takata, M., Hisataka, S., Hata, A., Inazu, T., Homma, Y., Nukaka, T., Yamamura, H. and Kurosaki, T. (1994) EMBO J. 13, 1341–1349.
[18] Oliver, J.M., Burg, D.L., Wilson, B.S., McLaughlin, J.L. and Geahlen, R.L. (1994) J. Biol. Chem. 269, 29697–29703.
[19] Weiss, A. and Littman, D.R. (1994) Cell 76, 263–274
[20] Reth, M. (1989) Nature 338, 383–384.
[21] Shiue, L., Zoller, M.J. and Brugge, J.S. (1995) J. Biol. Chem. 270, 10498–10502.
[22] Rowley, R.B., Burkhardt, A.L., Chao, H., Matsueda, G.R. and Bolen, J.B. (1995) J. Biol. Chem. 270, 11590–11594.
[23] Ben-Neriah, Yinon., Bernards, A., Paskind, M., Daley, G.Q. and Baltimore, D. (1986) Cell 44, 577–586.
[24] Burg, D.L., Furlong, M.T., Harrison, M.L. and Geahlen, R.L. (1994) J. Biol. Chem. 269, 28136–28142.
[25] Deng, W.P. and Nickoloff, J.A. (1992) Anal. Biochem. 200, 81–88.
[26] Kitts, P.A. and Posee, R.P. (1993) Biotechniques 14, 810–817.
[27] Peters, J.D., Furlong, M.T., Asai, D.J., Harrison, M.L. and Geahlen, R.L. (1996) J. Biol. Chem. 271, 4755–4762.
[28] Luo, K., Hurley, T.R. and Sefton, B.M. (1991) Methods Enzymol. 201, 149–152.
[29] Dadd, C.A., Cook, R.G. and Allis, C.D. (1993) Biotechniques 14, 266–273.
[30] Guan, K.L. and Dixon, J.E. (1991) Anal. Biochem. 192, 262–267.
[31] Taniguchi. T., Kobayashi, T., Kondo, J., Takahashi, K., Nakamura, H., Suzuki, J., Nagai, K., Yamada, T., Nakamura, S. and Yamamura, H., (1991) J. Biol. Chem. 266, 15790–15796.
[32] Benhamou, M., Ryba, N.J.P., Kihara, H., Nishikata, H. and Siraganian, R.P. (1993) J. Biol. Chem. 268, 23318–23324.
[33] Sillman, A. and Monroe, J.G. (1995) J. Biol. Chem. 270, 11806–11811.
[34] Aoki, Y., Kim, Y., Stillwell, U., Kim, T.J. and Pillai, S. (1995) J. Biol. Chem. 270, 15658–15663
[35] Kolanus, W., Romeo, C. and Seed, B. (1993) Cell 74, 171–183.
[36] Zioncheck, T.F., Harrison, M.L., Isaacson, C.C. and Geahlen, R.L. (1988) J. Biol. Chem. 263, 19195–19202.
[37] Couture, C., Baier, G., Oetken, C., Williams, S., Telford, D., Cardine, A.M., Baier-Bitterlich, G., Fischer, S., Burn, P., Altman, A. and Mustelin, T. (1994) Mol. Cell. Biol. 14, 5249–5258.
[38] Songyang, Z., Shoelson, M.C., Gish, G., Pawson, T., Haser, W.G., F. King., Roberts, T., Ratnofsky, S., Lechleider, R.J., Neel, B.G., Birge, R.B., Fajardo, J.E., Chou, M.M., Hana-

fusa, H., Schaffhausen, B. and Cantley, L.C. (1993) Cell 72, 767–778.
[39] Law, D.A., Chen, V.W.-F., Datta, S.K. and DeFranco, A.L. (1993) Curr. Biol. 3, 645–657.
[40] Kurosaki, T., Takata, M., Yamanashi, Y., Inazu, T., Taniguchi, T., Yamamoto, T. and Yamamura, H. (1994) J. Exp. Med. 179, 1725–1729.
[41] Rivera, V.M. and Brugge, J.S. (1995) Mol. Cell. Biol. 15, 1582–1590.
[42] Rowley, R.B., Bolen, J.B. and Fargnoli, J. (1995) J. Biol. Chem. 270, 18730–18733.
[43] Chan, A.C., Iwashima, M., Turck, C.W. and Weiss, A. (1992) Cell 71, 649–662.
[44] Watts, J.D., Affolter, M., Krebs, D.L., Wange, R.L., Samelson, L.E. and Aebersold, R. (1994) J. Biol. Chem. 269, 29520–29529.
[45] Zioncheck, T.F., Harrison, M.L. and Geahlen, R.L. (1986) J. Biol. Chem. 261, 15637–15643.
[46] Kobayashi, T., Nakamura, S., Taniguchi, T. and Yamamura, H. (1990) Eur. J. Biochem. 188, 535–540.
[47] Neumeister, E.N., Zhu, Y., Richard, S., Terhorst, C., Chan, A.C. and Shaw, A.S. (1995) Mol. Cell. Biol. 15, 3171–3178.
[48] Katsav, S., Sutherland, M., Packham, G., Yi, T. and Weiss, A. (1994) J. Biol. Chem. 269, 32579–32585
[49] Songyang, Z., Shoelson, S.E., McGlade, J., Olivier, P., Pawson, T., Bustelo, X.R., Barbacid, M., Sabe, H., Hanafusa, H., Yi, T., Ren, R., Baltimore, D., Ratnofsky, S., Feldman, R.A. and Cantley, L.C. (1994) Mol. Cell. Biol. 14, 2777–2785.
[50] Carter, R.H., Park, D.J., Rhee. S.G. and Fearton, D.T. (1991) Proc. Natl. Acad. Sci. 88, 2745–2749.
[51] Park, D.J., Rho, H.W. and Rhee, S.G. (1991) Proc. Natl. Acad. Sci. 88, 5453–5456.
[52] Kim, H.K., Kim, J.W., Zilberstein, J A., Margolis, B., Kim, C.K., Schlessinger, J. and Rhee, S.G. (1991) Cell 65, 435–441
[53] Seed, B., Kolanus, W., Romeo, C. and Xavier, R. (1994) in Mechanisms of Lymphocyte Activation and Immune Regulation V (Gupta, S., ed.), pp. 111–119, Plenum Press, New York.
[54] Law, C.L., Chandran, K.A., Sidorenko, S.P. and Clark, E.A. (1996) Mol. Cell. Biol. 16, 1305–1315.
[55] Hubbard, S.R., Wei, L., Ellis, L. and Hendrickson, W.A. (1994) Nature 372, 746–754.
[56] Iwashima, M., Irving, B.A., van Oers, N.S.C., Chan, A.C. and Weiss, A. (1994) Science 263, 1136–1139.
[57] Nada, S., Okada, M., Macauley, A., Cooper, J.A. and Nakagawa, H. (1991) Nature (London) 351, 69–72.